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Human protectin (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b–8 catalysed insertion of C9 into lipid bilayers

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SUMMARY

Human cells are relatively resistant to lysis by the homologous complement system. Here we describe the mechanism of action of a recently discovered and widely distributed 18,000–20,000 molecular weight (MW) membrane glycoprotein (CD59), which appears to act as a major protective element against complement-mediated lysis (hence called protectin). When incorporated into heterologous erythrocyte membranes, protectin efficiently prevented cell lysis by human serum. Neutralization with antibody of the naturally occurring protectin on human erythrocytes or on nucleated K562 cells increased their susceptibility to lysis by homologous complement. During complement activation, protectin became incorporated into the membrane attack complex (MAC). By interacting with newly exposed regions in the C5b–8 complex and in aggregating C9 it limited the number of C9 molecules associating with the C5b–8 complex to a C8:C9 ratio of 1:1·5 instead of a normal average of 1:3·5. The results demonstrate directly that protectin is a powerful inhibitor of complement cytolysis and acts by inhibiting the C5b–8 catalysed insertion of C9 into the lipid bilayer.

INTRODUCTION

The presence of the human CD59 antigen on blood leucocytes and erythrocytes has been substantiated by a group of monoclonal antibodies (mAb): MEM-43 (Stefanova *et al.*, 1989; IVth International Conference on Human Leukocyte Differentiation Antigens; Hadam, 1989), YTH53.1 (Davies *et al.*, 1989), H19 (Groux *et al.*, 1989) and 1F5 (Okada *et al.*, 1989b). The role of CD59 antigen as an inhibitor of membrane attack complex (MAC) was suggested by the observation that the YTH53.1 mAb enhanced reactive lysis of human erythrocytes by homologous complement (Davies *et al.*, 1989). Independent reports from other laboratories have also indicated that an 18,000–20,000 molecular weight (MW) glycoprotein acts as an inhibitor of the complement MAC (Sugita, Nakano & Tomita, 1988; Holguin *et al.*, 1989; Okada *et al.*, 1989a). Comparison of the different mAb and of the primary structure has demonstrated that CD59 is identical to this 18,000–20,000 MW complement lysis restricting factor and shows primary sequence homology (25%) with the mouse Ly-6 antigen family (Davies *et al.*, 1989; Okada *et al.*, 1989c). To clarify the wide array of names used: P-18 (an 18,000 MW MAC inhibiting factor; Sugita *et al.*, 1988), MIRL (membrane inhibitor of reactive lysis; Holguin *et al.*, 1989), HRF-20/1F5-ag (a 20,0000 MW homologous restriction factor; Okada *et al.*, 1989a), MEM-43-antigen (Stefanova *et al.*, 1989) or H19 (a 19,000 MW human erythrocyte molecule; Groux *et al.*, 1989; Whitlow *et al.*, 1990); and to implicate its protective function in complement lysis, we wish to propose the name 'protectin' for this molecule.

Thus far the molecular mechanism of action of protectin is unclear. Holguin *et al.* (1989) proposed that protectin, when incorporated into paroxysmal nocturnal haemoglobinuria (PNH) erythrocytes, inhibits the binding of complement components C7 and C8. In another set of studies (Okada *et al.*, 1989a; Whitlow *et al.*, 1990) it has been observed that the activity of protectin is strongest under conditions where C9 input is limiting. Similarities among the terminal complement components (C7, C8 and C9) themselves have led to suggestions that protectin may interfere with MAC assembly at multiple steps (Whitlow *et al.*, 1990). In the present paper we report the results of our studies aimed at elucidating the mechanism by which protectin inhibits MAC function.

Abbreviations: C, complement; C8bp, C8 binding protein; CVF, cobra venom factor; E, erythrocyte; EDTA, ethylene diamine tetraacetic acid; GPE, guinea-pig erythrocytes; GPI, glycosyl phosphatidyl inositol; HRF, homologous restriction factor; HSA, human serum albumin; LDH, lactate dehydrogenase; MAC, membrane attack complex; MIP, MAC inhibiting protein; NHS, normal human serum; PNH, paroxysmal nocturnal haemoglobinuria; SDS-PAGE, sodium dodecyl sulphate-polyacryl amide gel electrophoresis; TCC terminal complement complex.

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MATERIALS AND METHODS

Isolation of protectin

The erythrocyte membrane form of human protectin was purified from detergent solubilized human red cell ghosts by a modification of the described procedure (Davies *et al.*, 1989). Four units of packed human red cells were washed five times with phosphate-buffered saline (PBS), 10 mM azide (the last wash containing 5 mM EDTA, 10 mM benzamidine and 5 mM iodoacetamide to prevent proteolysis) and lysed in 1/10 ionic strength of the last washing buffer. Ghosts were washed extensively in a Pellicon cassette system (Cassette Durapore 00005 coarse filter, 0·45 μm; Millipore Ltd, Watford U.K.) prior to solubilization (overnight at +4°) in 2·0% Lubrol PX (Sigma Chemicals, St Louis, MO) detergent. After removal of the insolubilized material by centrifugation (25,000 *g*, 2 × 30 min), the supernatant was dialysed against half isotonic PBS containing 0·1% Lubrol PX detergent and subjected to YTH53.1–Sepharose affinity column chromatography. YTH53.1 is a rat anti-CD59 mAb (from G. Male, University of Cambridge), which has been characterized elsewhere (Davies *et al.*, 1989). Bound protectin was eluted from the column with a buffer containing 0·1 M acetic acid, 0·15 M NaCl and 0·1% Lubrol PX, pH 3·0, into tubes containing 0·5 M Tris buffer, pH 8·0, and dialysed against PBS containing 0·1% Lubrol. When necessary, remaining contaminants were removed by rechromatography on the YTH53.1 column or by gel filtration on the Sepharose 2B column. The final yield was approximately 0·7 mg of purified protectin (per batch of one litre of packed human red blood cells), which in SDS–PAGE (Laemmli, 1970) under non-reducing conditions gave a single band of approximately 18,000 MW.

Complement components and antisera

Cobra venom factor (CVF; Vogel & Müller-Eberhard, 1984), complement $C\overline{56}$ complexes (Lachmann & Thompson, 1970) and components C7, C8 and C9 were prepared as described elsewhere (Harrison & Lachmann, 1986; Morgan *et al.*, 1983; Abraha, Morgan & Luzio, 1988). Limited trypsin cleavage or α-thrombin cleavage of the component C9 was performed according to Shiver *et al.* (1986). Human perforin, partially purified from lymphokine-activated killer cells (Lichtenheld *et al.*, 1989), was a kind gift from Dr E. R. Podack (University of Miami School of Medicine).

Antisera against the complement components were obtained by immunizing rabbits with isolated components (Harrison & Lachmann, 1986). Anti-C7, -C8 and -C9 antisera were also purchased from Serotec (Oxford, U.K). IgG purification and generation of the Fab_2 fragments was as described previously (Davies *et al.*, 1989). Proteins were radiolabelled with ^{125}I or ^{131}I using the Iodogen method (Pierce Chemical Co., Rockford, IL).

Lysis assays

The reactive lysis of human erythrocytes (Lachmann & Thompson, 1970) was achieved by mixing 2×10^7 human red blood cells with increasing concentrations of anti-protectin and control antibodies (5 min at +37°) and incubating the cells with $C\overline{56}$ (88 μg/ml) and EDTA plasma (final 12·5%) for 30 min at +37°. Haemolysis was calculated on the basis of haemoglobin release by absorbance at 412 nm and expressed as a percentage of total lysis after subtraction of background lysis (lysis in the absence of added $C\overline{56}$). Killing of nucleated (K562 or HeLa) cells was performed similarly, except that higher concentrations of EDTA plasma or serum (up to 50%) were used. Alternatively, nucleated cells (or erythrocytes) were lysed by CVF-mediated complement lysis by incubating cells in the presence of 25 μg/ml CVF and appropriate dilutions of normal human serum (NHS). Lysis of nucleated cells was estimated by release of lactate dehydrogenase (LDH; Morgan, Dankert & Esser, 1987).

Incorporation of ^{125}I-protectin into cell membranes

Purified protectin was incorporated into membranes of guinea-pig (GPE), rabbit, mouse, sheep or chicken erythrocytes (E) by incubating 10^8 cells/tube for 1 hr at +37° with increasing amounts of ^{125}I-labelled protectin in a final volume of 200 μl of TBS (0·05 M Tris-HCl, 0·15 M NaCl, pH 7·4). The amount of detergent (Lubrol PX) in the protectin preparation was reduced to a level below 0·001% by using microconcentration and dialysis devices from Amicon (Danvers, MA) prior to incubation with erythrocytes. Cells were washed twice with TBS and the radioactivity was measured. The amount of protectin incorporated was calculated on the basis of its known specific activity value. Incorporation of protectin into HeLa or K562 cells was performed with or without pretreatment of the cells with the YTH53.1 antibody (to neutralize the existing protectin molecules on the cell membrane). For antibody treatment, cells were incubated for 15 min at +30° with 50 μg/ml of Fab_2 fragments of the YTH53.1 antibody. Washed cells in RPMI were treated with 50 μg/ml of the membrane form of protectin or with an equivalent amount of dialysis medium (15 min at +30°) prior to lysis assays.

Neutralization of the incorporated protectin was achieved by incubating 10^7 GPE/tube carrying approximately 5000, 1600 or 0 (control) protectin molecules/cell with the YTH53.1 or YTH89.1 (G. Male) IgG (10 min at +37°). Cells were subjected to reactive lysis ($A_{412\ nm}$) by adding $C\overline{56}$ (2·6 μg) and EDTA plasma (final 12%) and incubating for 30 min at +37° in 130 μl PBS.

Estimation of the number of protectin molecules/erythrocyte

Human erythrocytes (in triplicate) and control cells (sheep erythrocytes) were incubated for 30 min at +37° with increasing concentrations of the ^{125}I-labelled YTH53.1 IgG. Bound antibody was separated from free by centrifugation through oil in microcentrifuge tubes. For determination of the number of protectin molecules it was assumed that binding was monovalent at saturating levels of the antibody.

Preparation of cell intermediates and analysis of stage of protectin action

Sera selectively depleted of each of the terminal C components (C7DS, C8DS and C9DS) were prepared by affinity chromatography using columns with specific mAb (anti-C7 , anti-C8 and anti-C9) covalently coupled to Sepharose (Pharmacia, Uppsala, Sweden; Morgan *et al.*, 1983; Abraha *et al.*, 1988). Sera were also depleted of S protein (or vitronectin) using anti-S protein monoclonal IgG coupled to Sepharose (Hodgetts & Morgan, 1989). The red cell (GPE) intermediate EC5b–7 was prepared by using CVF (25 μg/ml) and NHS (diluted 1/10 in GVB^{2+}) depleted of C8, C9 and of S protein (NHS_{depl}). EC5b–8 was obtained using a similar treatment with NHS_{depl} reconstituted with C8 (10 μg/ml). EC5b–9 cells were obtained by treating EC5b–8 cells with isolated C9 (10 μg/ml) for 30 min at 0° (C5b–9; Bhakdi & Tranum-Jensen, 1986). Protectin (approximately 2600 molecules/cell) was incorporated into GPE at

various stages of MAC formation: (i) prior to treatment with CVF-NHS_{depl} and reconstitution with C8 and C9; (ii) prior to C8 and C9 reconstitution (C5b–7); (iii) prior to C9 reconstitution (C5b–8); or (iv) after C5b–8 formation and incubation with C9 at 0°. For (i) to (iii), protectin was incorporated by incubation for 5 min at +37°, and for (iv) for 15 min at +4° (preliminary experiments having demonstrated incorporation at +4°). Lysis of the various intermediates and their controls without incorporated protectin was determined as haemoglobin release (A_{412} nm) after a 30-min incubation at +37°.

Protectin-induced inhibition of the lytic activity of trypsin- or α-thrombin-treated C9 was studied by using GPE carrying C5b–8 complexes (as in stage iii). Cells were treated with protectin or with GVB (control) and native or cleaved C9 (0·5 mg/ml) was added at increasing amounts into the cell suspensions. Lysis was determined after a 30-min incubation at +37° by measuring the absorbance at 540 nm.

Gel filtration of MAC

GPE (10^6 cells/tube) carrying approximately 2500 ^{125}I-labelled protectin molecules/cell were incubated (1 hr at +37°) with CVF (25 μg/ml) and with either NHS, C7DS, C8DS or C9DS. Cells were lysed in H_2O and membranes solubilized in TBS containing 1% CHAPS (Sigma Chemical Co., St Louis, MO) in a final volume of 1 ml. The solubilizates were subjected to Sepharose 2B (1·5 × 20 cm, total volume 35 ml; Pharmacia) chromatography using veronal-buffered saline (VBS), CHAPS 0·1% as buffer. Fractions (0·5 ml) from each membrane preparation were collected in a similar manner and counted for radioactivity. Cells without serum treatment were taken as controls for non-complex associated ^{125}I-protectin on the membrane. The terminal C complexes forming were identified in separate, but identical, experiments using sera containing radiolabelled C8 or C9.

Sucrose density gradient analysis

NHS (100 μl) or sera depleted of C7, C8 or C9 containing ^{125}I-protectin were activated by CVF (25 μg/ml, 45 min at +37°) or by inulin (4% w/v), activation stopped with 20 mM EDTA and mixtures layered on top of 10–50% sucrose gradients in PBS, 0·1% NP40 (Sigma) (or 0·1% Lubrol PX). Gradient tubes were centrifuged for 14–20 hr at 300,000 *g* using a SW65 rotor in a Centrikon T-2070 ultracentrifuge (Kontikon Instruments, Zürich, Switzerland). Fractions (150 μl) were collected from the bottom of the tubes and counted for radioactivity. To localize the assembled terminal complement complexes (TCC) ^{131}I-C8 or ^{131}I-C9 were added to NHS prior to activation. Protein concentration in the fractions was determined by using the BioRad dye test (BioRad, Richmond, CA). Non-activated serum with ^{131}I-C8 and ^{125}I-protectin was used as control and haemoglobin (Hb), IgG and IgM as migration markers.

To test complex formation of protectin with individual C components, purified human $C\overline{56}$, C7, C8, C9 or controls [perforin, human serum albumin (HSA)] were mixed at varying concentrations (range from $0{\cdot}66 \times 10^{-7}$ M to $1{\cdot}4 \times 10^{-6}$ M) with ^{125}I-labelled protectin ($0{\cdot}59 \times 10^{-7}$ M), incubated for 1 hr at +37° in PBS, 0·1% NP40 and layered on top of a 10–50% sucrose gradient in PBS, 0·1% NP40. The gradients were ultracentrifuged for 22 hr at 500,000 *g* at +20°.

Spontaneous polymerization of C9 was tested by incubating C9 (7 μM) with or without $ZnCl_2$ (50 μM) at +37° for various times (up to 72 hr; Tschopp, 1984) in the presence or absence of ^{125}I-labelled protectin (3·5 μM). Polymers were separated by sucrose gradient centrifugation or by gel filtration and visualized by SDS–PAGE analysis.

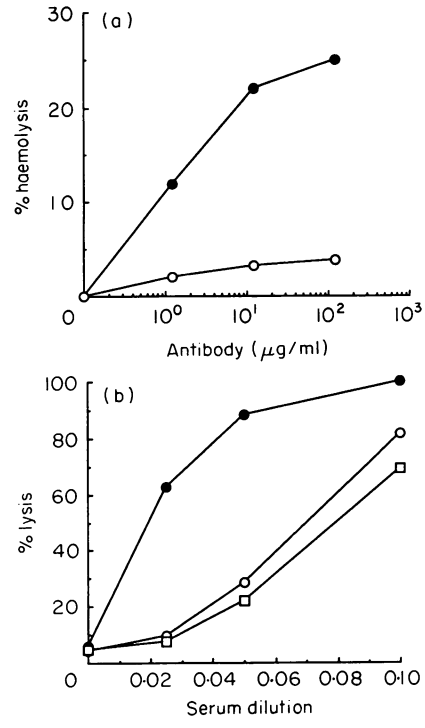

Figure 1. The effect of the monoclonal anti-protectin antibody on reactive lysis of human erythrocytes (a) or on complement killing of nucleated K562 cells (b). In (a) human red blood cells were incubated with increasing amounts of the anti-protectin (YTH53.1) (●) and control (YTH89.1, anti-glycophorin A) (○) antibodies and subjected to reactive lysis by human terminal C components ($C\overline{56}$ + EDTA plasma as a source for C7, C8 and C9). In (b) the human erythroleukaemia K562 cells were subjected to complement lysis by treating the cells with varying dilutions of human serum and CVF (25 μg/ml) in the presence (10 μg/ml) of the YTH53.1 (●) or YTH89.1 (○) antibody or non-immune mouse IgG (□). Cell lysis was determined either as haemoglobin (a) or lactate dehydrogenase (LDH) release (b) after a 30 min incubation at +37°.

RESULTS

Enhancement of reactive lysis by anti-protectin antibodies

When normal human erythrocytes or nucleated K562 cells were treated with the mAb YTH53.1 their sensitivity to reactive (E) or CVF-NHS-mediated (K562) lysis significantly increased (Fig. 1). This effect increased linearly as a response to increased doses of either antibody or complement. A control antibody (anti-glycophorin A) of the same isotype as YTH53.1 (rat IgG2b) caused little enhancement of lysis in either case.

Enumeration of protectin molecules on erythrocytes

Using the IgG fraction of the ^{125}I-labelled YTH53.1 antibody, the average number of protectin molecules on human erythrocytes was estimated. After correction for non-specific binding it was calculated that, at saturation, 638 ng IgG/$1{\cdot}0 \times 10^8$ human

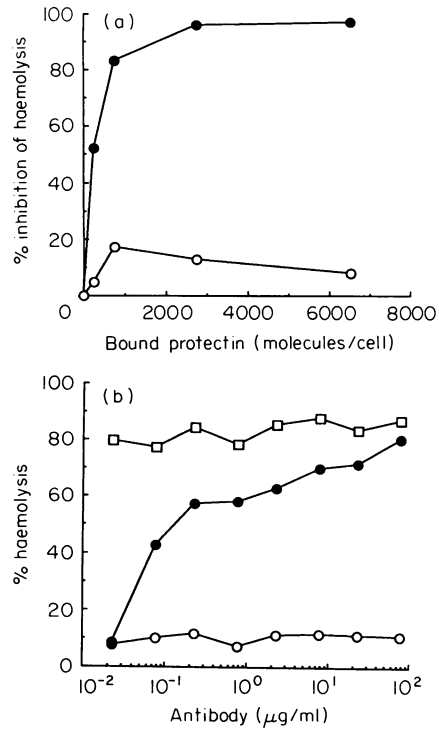

Figure 2. The effect of protectin incorporation on the sensitivity of heterologous erythrocytes to complement-mediated lysis (a) and neutralization of incorporated protectin by an anti-protectin monoclonal antibody (b). (a) Guinea-pig (●) or sheep (○) erythrocytes ($1{\cdot}25 \times 10^6$ cells/tube) carrying increasing concentrations of incorporated human protectin were subjected to reactive lysis by incubating them for 10 min at +37° with 8·4 μg $C\overline{56}$ and human EDTA plasma (25%). The effect of protectin incorporation is expressed as percentage inhibition of initial haemolysis ($A_{412\,nm}$) in the absence of incorporated protectin. (b) GPE (10^7 cells/tube) with (○, ●) or without (□) incorporated protectin (5000 molecules/cell) were treated (10 min) with increasing concentrations of the YTH53.1 anti-protectin antibody (□, ●) or with the YTH89.1 control (anti-glycophorin A) (○) antibody and subjected to reactive lysis (30 min at +37°) by $C\overline{56}$ (20 μg/ml) and EDTA plasma (12%, final). Haemolysis was determined as absorbance at 412 nm.

red blood cells were specifically bound. This corresponds to $24{\cdot}7 \times 10^3$ IgG molecules per cell and gives an estimate of 25,000 protectin molecules per human red cell.

Restriction of complement lysis after incorporation of protectin into heterologous red cell membranes

Purified ^{125}I-labelled protectin was incorporated into membranes of heterologous red cells from four different species: guinea-pig, rabbit, mouse and sheep. The uptake of ^{125}I-protectin in each case ranged from 14% to 33% and did not significantly vary between cells from different species. Maximally, approximately 6500 protectin molecules/cell could be incorporated.

In three out of the four species (guinea-pig, rabbit and mouse) studied, a similar dose–response increase in protection against lysis by human complement was achieved after incorporating protectin into the cells. GPE are shown as an example in Fig. 2a. On the basis of the specific activity of the radiolabelled and cell-bound protein, it was calculated that a level of 50% protection was achieved when on the average 250 molecules per cell were incorporated.

Table 1. Stage of complement lysis inhibition by protectin

Stage	% lysis		% inhibition
	No protectin	With protectin	
Pre-C7	33	4	88
Pre-C8	62	18·5	70
Pre-C9	49	12·5	74
Pre-lysis	77	66	14

Red cell intermediates carrying various complement components were prepared by using CVF and NHS depleted of S protein, C8 and C9 (NHS_{depl}). Protectin (approximately 2600 molecules/cell) was incorporated into GPE (i) prior to treatment with CVF-NHS_{depl} and reconstitution with C8 and C9; (ii) prior to C8 and C9 reconstitution; (iii) prior to C9 reconstitution; or (iv) after C5b–8 formation and incubation with C9 (30 min) at 0°. Lysis of the various intermediates and their controls without incorporated protectin was determined as haemoglobin release (A_{412} nm) after a 30-min incubation at +37°. Results are the means of triplicate measurements and are representative of four separate experiments.

Sheep erythrocytes were more resistant to reactive lysis using human complement (C) components than cells from the three other species. This naturally higher resistance against lysis by human complement could be overcome when the amount of offered complement was increased (by approximately 50%). Under conditions where sheep erythrocytes were lysed by human complement, incorporated protectin did not significantly inhibit lysis (Fig. 2a). Thus human protectin did not have the same activity on sheep cells as on the other tested erythrocytes, although the amounts of incorporated protectin were comparable. When sheep erythrocytes were treated with the YTH53.1 antibody, no specific binding and no enhancement of reactive lysis by human complement was observed.

Neutralization of incorporated protectin by YTH53.1

To see if the incorporated protectin could be fully neutralized, GPE, carrying an average of 5000 protectin molecules/cell, were treated with increasing doses of the YTH53.1 or of control (YTH89.1) antibodies and subjected to reactive lysis. As can be seen from Fig. 2b the resistance of GPE with incorporated protectin to reactive lysis could be fully reversed with the monoclonal YTH53.1, but not with the control YTH89.1 antibody. When GPE carrying an average of 1500 protectin molecules/cell were tested similarly, the point where 50% of cells were lysed was reached using approximately threefold less antibody.

Analysis of the stage of protectin action using cell intermediates

The ability of the YTH53.1 antibody to enhance C5b–9-mediated reactive lysis suggested that protectin exerted its inhibitory effect at the terminal stage of complement lysis. To locate the interaction site more specifically, protectin was

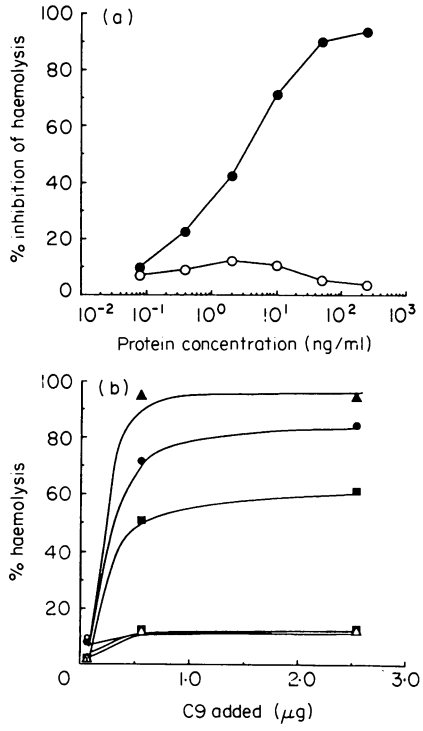

Figure 3. The effect of protectin incorporation on GPE lysis after formation of the C5b–8 complex (a) and on GPE lysis by proteolytically cleaved C9 (b). In (a) the C5b–8 complex was assembled from isolated components $C\overline{56}$, C7 and C8 and complement lysis was developed by adding C9. Inhibition of lysis was assessed in the presence of human protectin (●) or BSA (control) (○), that were offered in PBS containing 0·004% of Lubrol PX detergent. In (b) GPE were incubated with C9-depleted serum and CVF for 15 min at +37° prior to incorporation of protectin into the cell membranes. Lysis was detected as haemoglobin release by absorbance at 540 after adding C9 (circles), trypsin-treated C9 (squares) or α-thrombin-treated C9 (triangles) to the mixtures with (empty symbols) or without (filled symbols) protectin.

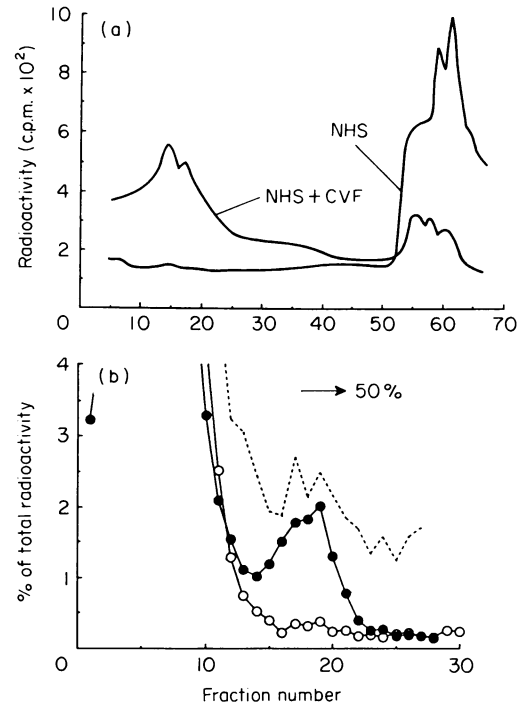

Figure 4. Interaction of protectin with the C MAC (a) and with the fluid-phase TCC (b). (a) ^{125}I-labelled protectin was incorporated into GPE and portions of the cells were incubated with CVF (25 μg/ml) and NHS (30 min at +37°) or with only NHS as a control. Membranes were extracted with detergent and run on Sepharose 2B gel filtration column in VBS, 0·1% CHAPS. Comparison of the elution pattern between complement-lysed and unlysed membranes revealed a shift in the migration pattern of ^{125}I-protectin on lysed cells. The larger molecular weight peak that was induced by complement lysis (left) co-migrated with ^{125}I-C8 and ^{125}I-C9 on identical but separate runs, indicating formation of MAC on the membrane and incorporation of protectin into it. (b) Incorporation of protectin into the fluid-phase TCC. NHS containing ^{131}I-C8 (- - -) and ^{125}I-protectin (●) was activated by CVF and analysed for formation of TCC by sucrose density gradient analysis. Comparison with non-activated control (NHS with ^{131}I-C8 and ^{125}I-protectin without CVF treatment) (○) showed the appearance of the TCC upon activation, where both ^{131}I-C8 and ^{125}I-protectin were incorporated.

incorporated into red cell intermediates carrying C5b–7, C5b–8 or C5b–9 complexes (Table 1). The intermediates were prepared by using GPE and human serum that was originally depleted of S protein (to enhance site formation), C8 and C9 and subsequently reconstituted with C8 plus or minus C9. For effective intermediate C complex formation, serum was activated with CVF, and lysis after protectin incorporation developed by adding the remaining components. To obtain assembly of C5b–9 without lysis, the last component C9 was added at 0° and lysis subsequently developed at +37°. As shown in Table 1, the analysis indicated that protectin acted at a stage beyond formation of the C5b–8 complex, i.e. protectin was capable of inhibiting lysis after the C5b–8 complex had become assembled ('pre-C9' stage), but not after C9 had been allowed to bind to the complex ('pre-lysis' or the S* stage).

When protectin was incorporated into GPE at the 'pre-C9' stage using isolated $C\overline{56}$ complexes and C7 plus C8, a clear dose-response inhibitory effect on lysis by subsequently added C9 was documented (Fig. 3a). C9 cleaved with trypsin or α-thrombin (Shiver *et al.*, 1986) maintained full cytolytic activity. Incorporation of protectin into GPE after formation of C5b–8 caused inhibition of subsequent lysis by both trypsin and α-thrombin-treated C9, which was equivalent to that seen with native C9 (Fig. 3b).

Incorporation of ^{125}I-protectin into MAC and TCC

To analyse the possible interaction of protectin with the complement MAC, ^{125}I-protectin was incorporated into GPE, which were then lysed with CVF plus NHS. After solubilization of the membrane and gel filtration analysis (Fig. 4a), the radiolabel was observed to become associated with a high molecular weight complex that co-migrated with radiolabelled C8 or C9. An analogous experiment in the fluid phase using sucrose density gradient centrifugation analysis (Fig. 4b) indicated association of ^{125}I-protectin with the soluble SC5b–9 terminal complex (TCC) that was formed during C activation in serum.

When the C membrane complexes were generated in C8-depleted serum to form SC5b–7, no association of ^{125}I-protectin

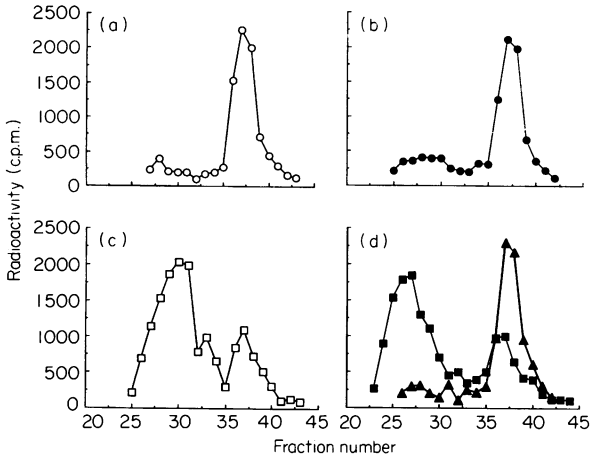

Figure 5. Incorporation of protectin into membrane-associated C5b-8 complexes. ^{125}I-protectin was incorporated into GPE membranes, as in Fig. 4a. Cells were treated with C7-(a), C8-(b) or C9-(c) depleted or normal (d) serum that were activated with CVF (25 μg/ml). In (d) the control included only ^{125}I-protectin incorporated GPE+CVF. After extraction of the membranes into detergent and gel filtration, protectin can be observed to have become incorporated into membrane C5b-8 (c) and C5b-9 (d), but not into C5b-7 complexes (b). (○) C7DS; (●) C5b-7; (□) C5b-8; (■) C5b-9; (▲) control.

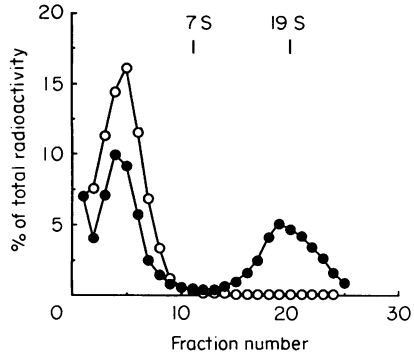

Figure 6. Incorporation of protectin into fluid-phase SC5b-8 complexes. ^{125}I-protectin was added into C8- (○) or C9-depleted (●) serum prior to activation with inulin and the mixtures were subjected to sucrose density gradient centrifugation analysis.

with the complex was observed. When membrane C5b-8 complexes were formed using C9-depleted serum a clear association of ^{125}I-protectin with the generated complex could be detected as a shift in the migration pattern (Fig. 5).

Similarly, ^{125}I-protectin was observed to become incorporated into the fluid-phase SC5b-8 complex (Fig. 6) during C activation by CVF or inulin. When analysis was performed using serum depleted of C7 or C8, no shift in the mobility of ^{125}I-protectin was observed indicating a lack of ^{125}I-protectin association with the fluid phase $C\overline{56}$ and C5b-7 complexes.

Analysis of the effect of protectin on C8:C9 ratio in the MAC

To evaluate the amount of C9 incorporated into the forming MAC in the presence of protectin, comparative binding studies

Table 2. The effect of protectin on relative molecular ratios of C8 and C9 bound to MAC

	No protectin	With protectin
C8 bound	1	1
C9 bound	3·3	1·5

Protectin treated or untreated GPE were incubated with CVF (25 μg/ml) and either C8-depleted serum + ^{125}I-C8 or C9-depleted serum + ^{125}I-C9. Control cells were incubated similarly but without CVF. Unlysed cells were washed and the specific binding of C8 and C9 calculated on the basis of their known specific activities. Numbers indicate the molar ratio of binding relative to C8 binding. Results are the means of triplicate determinations.

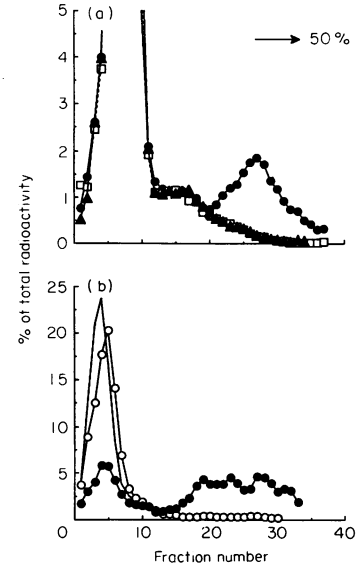

Figure 7. The interaction of protectin with individual fluid-phase C components. (a) Purified human C components (C7, 1×10^{-7} M, ▲; C8, $0{\cdot}89 \times 10^{-6}$ M, □; C9, $1{\cdot}4 \times 10^{-6}$ M, ●) were mixed with ^{125}I-labelled protectin ($0{\cdot}59 \times 10^{-7}$ M), incubated for 1 hr at +37° in PBS, 0·1% NP40, and layered on top of a 10–50% sucrose gradient in PBS, 0·1% NP40. The gradient tubes were ultracentrifuged for 22 hr at 500,000 *g* at 20°. (b) Interaction of ^{125}I-protectin with C9 at low ionic strength. C8 (○) or C9 (●) (both at 1×10^{-6} M) were incubated with ^{125}I-protectin and subjected to sucrose density gradient centrifugation at 1/10 PBS, 0·01% Lubrol PX. (——) Protectin alone.

using C8-depleted serum + ^{125}I-C8 and C9-depleted serum + ^{125}I-C9 were carried out (Table 2). It was found that the presence of protectin on the membrane restricted the number of inserted C9 molecules to an approximate molar C9/C8 ratio of 1·5 instead of an average normal ratio of 3·3. Thus two to three times less C9 is incorporated into MAC in the presence of protectin.

According to Bhakdi & Tranum-Jensen (1986), only one C9 molecule binds per C5b-8 site at +0°, and only becomes

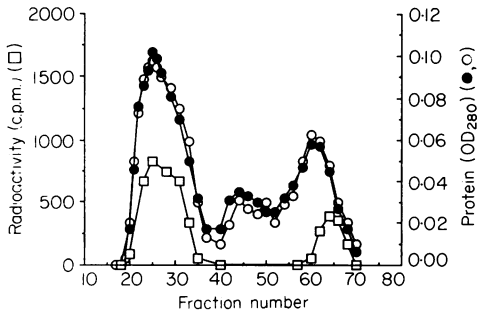

Figure 8. Incorporation of protectin into poly-C9. Human C9 (7 μM) was polymerized by incubation (24 hr) with $ZnCl_2$ (50 μM) at +37° in the presence (●) or absence (○) of ^{125}I-labelled protectin (3·5 μM). After incubation, mixtures were applied to a Sepharose 2B gel filtration column using TBS, pH 7·4, as buffer. Radioactivity was measured in the eluted fractions (0·5 ml) and protein content followed by absorbance at 280 nm.

incorporated upon warming to 37°. Prior to warming, the non-inserted ^{125}I-C9 is displaceable by unlabelled C9 (Morgan, 1984). When an attempt was made to displace ^{125}I-C9 on cells bearing protectin no such displacement occurred, suggesting that the ^{125}I-C9 bound in the presence of protectin was more firmly incorporated into the MAC than that bound at 0° (data not included).

Interaction of ^{125}I-protectin with isolated C components in the fluid phase

Direct interactions of protectin with individual human C components or perforin were studied by density gradient centrifugation analysis after mixing and incubating ^{125}I-protectin with $\overline{C56}$, C7, C8, C9, perforin or HSA. The results (Fig. 7) showed that protectin formed a complex with C9 but not with the other tested molecules. The sedimentation profiles of ^{125}I-protectin with $\overline{C56}$, perforin and HSA were similar to those shown for C7 and C8. Binding of protectin to C9 was dose-dependent, reaching saturation at an approximate C9:protectin molar ratio of 25:1. Using a highly purified ^{125}I-protectin preparation, about 80% of the radiolabel was associated with high molecular weight complexes of C9. At low ionic strength (1/10 PBS) and at lower concentrations of detergent (0·01% Lubrol PX) in the gradients a heterogeneous pattern in complex formation was observed (Fig. 7b). The formation of the apparently larger molecular weight complexes suggested binding to oligomerized C9 present (or formed during the incubation) in the C9 preparation rather than to native C9.

The absence of complex formation with fluid-phase C8 was verified with two separate C8 preparations and using molar C8:protectin ratios of up to 30:1. Also, no C8 complexes were detected when sucrose gradients were run under conditions (low ionic strength, $\mu = 0{\cdot}015$), which would favour weak interactions.

Lack of inhibition of spontaneous C9 polymerization by protectin

To determine whether or not protectin is capable of preventing spontaneous C9 polymerization, C9 was polymerized by prolonged incubation at +37° or in the presence of Zn^{2+} ions (50 μM) in the presence or absence of protectin (Tschopp, 1984). When polymerized C9 was separated from monomeric C9 by gel filtration no difference in the extent of poly-C9 formation was observed (Fig. 8), and the appearance of the poly-C9 on SDS-PAGE was unaltered by inclusion of protectin. However, when C9 was incubated with Zn^{2+} ions in the presence of ^{125}I-protectin, the radiolabel was observed to become incorporated into the generated poly-C9 complexes (Fig. 8). Some binding of ^{125}I-protectin occurred also to premade poly-C9, but the amount was less than 10% of that when ^{125}I-protectin was present during the incubation. No complex formation of ^{125}I-protectin occurred during incubation with zinc alone.

DISCUSSION

In this study we have delineated the stage and, at least to some extent, the mechanism of action of the MAC-inhibiting protein protectin (CD59). Protectin acts at the final stages of MAC assembly and inhibits by limiting C9 input into the forming complex. The protein incorporates into C5b-8, C5b-9 and poly-C9, but not into C5b-7 complexes, suggesting an interaction with complex-associated C8 and C9.

To clarify the stage at which protectin interacts with the assembly of MAC, protectin was incorporated into cell membranes after generation of various intermediate MAC complexes. Addition of protectin at any stage up to and including EC5b-8 efficiently inhibited lysis upon subsequent addition of the terminal components. However, addition of protectin after binding of C9 to EC5b-8 at zero did not inhibit lysis during subsequent incubation at 37°. These experiments imply that protectin interacts with the forming MAC at the stage where the first C9 molecule is becoming bound to the C5b-8 complex. Once a single C9 has bound to C5b-8 the addition of protectin does not inhibit lysis.

Studies using radiolabelled protectin clearly demonstrated that it became incorporated into the MAC at the C5b-8 stage, remaining associated with C5b-8 and C5b-9 complexes even following detergent extraction of the membrane. No association with C5b-7 complexes was detectable. Similarly, protectin incorporated into fluid-phase SC5b-8 and SC5b-9, but not into SC5b-7, complexes. These findings, together with the demonstration that protectin does not bind to C8 in the fluid phase, suggest that in the C5b-8 complex protectin binds to a 'neoepitope' not present in the native components, but exposed upon C8 incorporation. Although it is likely that this binding site is on C8, the possibility remains that protectin binds elsewhere in the complex to a site which is only exposed following C8 binding. The binding site for C9 in the C5b-8 complex is on the α-chain of C8 (Stewart & Sodetz, 1985). Addition of protectin after binding of a single C9 molecule to the forming complex does not inhibit lysis; although we have not yet demonstrated that loss of inhibition is due to blocking of the binding of protectin, this finding again implicates C8, and specifically the α-chain, as the binding site.

In the presence of protectin, C5b-8 complexes bind an average of only 1·5 C9 molecules, whereas in its absence, in agreement with previous estimates (Sims, 1983; Stewart *et al.*, 1984), an average of 3·5 C9 molecules is bound. These results suggest that protectin inhibits MAC activity by limiting the input of C9. The presence of single, rather than multiple, C9 molecules per complex is not in itself sufficient to explain the inhibition of lytic activity. Incubation of C5b-8-coated cells

with C9 at 0° allows binding of only a single C9 molecule per complex, yet on subsequent warming to 37° a functional lesion is formed (Bhakdi & Tranum-Jensen, 1986). The single C9 molecule bound in the presence of protectin, unlike that bound at 0°, must therefore either be prevented from being inserted or be inserted in a functionally inactive form, thereby avoiding membrane disruption. Whereas labelled C9 bound at 0° was, prior to the temperature-dependent 'insertion' step, displaceable by unlabelled C9, binding in the presence of protectin was not displaceable. Therefore the single C9 molecule has become firmly incorporated into the C5b–8–protectin complex instead of merely being loosely bound. This incorporation, however, must occur in a fashion which does not disrupt the membrane. Thus, in addition to inhibiting C9 input into C5b–8 complexes, protectin appears to block the functional activity of the incorporated C9 in the C5b–9 complex.

In binding studies using sucrose density gradient analysis protectin was found to interact with C9 oligomers, but not with any native terminal C components (or with perforin). C9 polymerization by zinc was not quantitatively affected by the presence of protectin, but incorporation of protectin into the poly–C9 complex was observed. C9 can be modified by limited digestion with trypsin or by cleavage with α-thrombin to yield a molecule which, while retaining full haemolytic activity, is incapable of forming ring polymers (Dankert & Esser, 1985). Protectin incorporation into EC5b–8 efficiently inhibited lysis by these modified forms of C9, confirming that inhibition was not mediated merely by influencing ring polymer formation.

Incorporation of protectin rendered erythrocytes from all species tested, with the exception of sheep, resistant to subsequent lysis by the human MAC. Compared to other heterologous red cells sheep erythrocytes are relatively resistant to lysis by human C components in a reactive lysis system. It is possible that the failure of protectin to protect sheep erythrocytes is due to the larger numbers of MAC needed for lysis. However, sheep cells also show homologous restriction (which extends also to horse complement) and could thus have an analogous 'protectin'. This may be the molecule recently described in sheep T cells (Hein & Beya, 1989). It is therefore possible that the protection afforded to sheep red cells also extends to human complement.

Although the CD59 antigen has been identified on a wide range of nucleated cells (Davies *et al.*, 1989), evidence of MAC inhibitory function had previously only been described on erythrocytes and platelets (Sugita *et al.*, 1988; Holguin *et al.*, 1989; Sims, Rollins & Wiedmer, 1989). In this study we have provided evidence that the CD59 antigen on the nucleated cell line K562 is also functionally relevant, neutralization of CD59 with antibody rendering the cells increasingly susceptible to lysis by homologous complement. Preliminary evidence indicates that the CD59 antigen on a variety of other nucleated cells also plays an important role in limiting C lysis (I. Rooney, D. Griffiths, J. Williams, S. Meri, P. J. Lachmann and B. P. Morgan, manuscript submitted for publication).

Current models of MAC assembly envisage the C5b–8 complex acting as a receptor for C9 and as an accelerator of its polymerization. Binding of C9 to a high-affinity site on C5b–8 initiates C9 unfolding and triggers C9 polymerization (Podack, 1988; Stanley, 1988). The mechanisms by which unfolding and polymerization of C9 proceed are unknown, but it has been suggested that disulphide-rich areas exposed in unfolded C8 and C9 (and in C6 and C7) act as intermolecular 'fasteners',

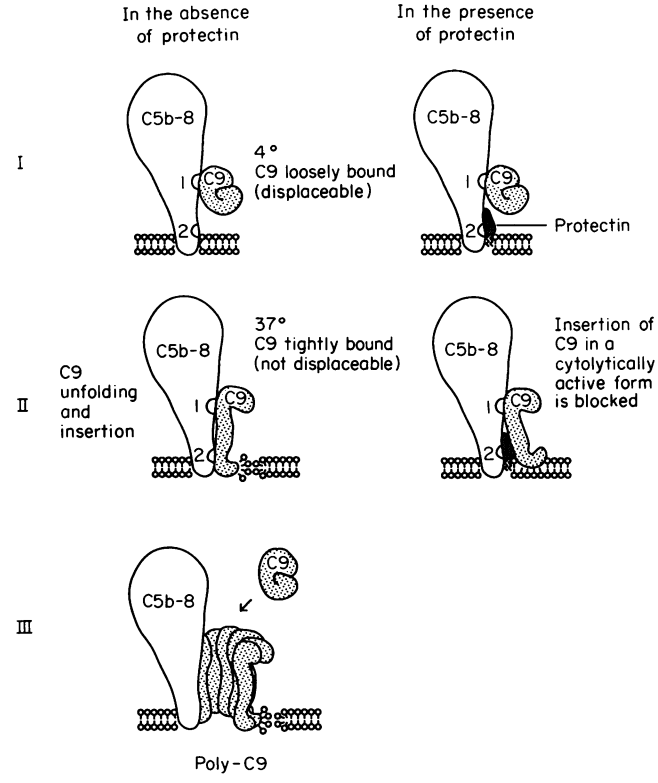

Figure 9. Schematic representation of the proposed mechanism of protectin function. In this model we envisage a minimum of two hypothetical binding sites on C5b–8 for C9 (sites 1 and 2). At 4° C9 becomes loosely bound at site 1, and may be displaced (Stage I). Upon warming to 37° C9 starts unfolding, during which it interacts with a second site in C5b–8 and becomes integrated in the membrane (Stage II). Membrane damage ensues following exposure of (partly hydrophobic) new interaction sites with the lipid bilayer and by formation of transmembrane channels (Stage III). In the presence of protectin the initial binding of C9 to C5b–8 occurs as in Stage I, but the subsequent insertion of C9 and interaction with site 2 on C5b–8 is prevented. Inhibition of the interaction between C5b–8 and C9 is believed to be due to blocking of site 2 on C5b–8 by protectin. The firm association of the 'first' C9 molecule with C5b–8–protectin complex could be achieved by the interaction of protectin with C9 whereby the recruitment of additional C9 molecules into the complex is prevented.

sequentially locking the molecule in its new conformation as it unwinds (DiScipio *et al.*, 1988). From the data presented here we propose that binding of protectin to C5b–8 prevents the expression of or blocks one or more of the sites to which C9 attaches during the unwinding process. A single molecule of C9 thus still binds to its unblocked attachment site and perhaps undergoes the initial stages of unwinding, rendering it more firmly attached and undisplaceable. Blocking of later binding sites by protectin prevents the full unwinding of C9, and hence the molecule does not become inserted or expose the sites necessary for binding and unwinding of the next C9 in the sequence of assembly (Fig. 9). Although not yet demonstrated, it is possible that even if one C9 molecule has incorporated prior to interaction of the complex with protectin, binding to the equivalent sites on this C9 molecule will prevent further recruitment of C9, limiting the size of the lesion. The demonstration that protectin did not inhibit spontaneous or Zn^{2+} induced C9 polymerization *in vitro* despite its incorporation into the

complex implies either that different interaction sites are involved in this process or that in this artificial situation and in the presence of a large molecular excess of C9 the inhibitory action of protectin can be overcome.

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